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Determination of ochratoxin A in maize bread samples by LC with fluorescence detection

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Abstract

Ochratoxin A (OTA) is a secondary fungal metabolite produced by several moulds, mainly by *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and by *Penicillium verrucosum*. The present work shows the results of comparative studies using different procedures for the analysis of OTA in maize bread samples. The studied analytical methods involved extraction with different volumes of PBS/methanol, different extraction apparatus, and clean-up through immunoaffinity columns. The separation and identification were carried out by high-performance liquid chromatography with fluorescence detection. The optimized method for analysis of OTA in maize bread involved extraction with PBS:methanol (50:50), and clean-up with IAC column. The limit of quantification was 0.033 ng g^{-1}. Recoveries ranged from 87% to 102% for fortifications at 2.000 and 0.500 ng g^{-1}, respectively, within-day R.S.D. of 1.4% and 4.7%. The proposed method was applied to 15 samples and the presence of OTA was found in nine samples at concentrations ranging from nd to 2.650 ng g^{-1}.

Keywords: Ochratoxin A; Maize bread; LC

1. Introduction

OTA is mainly produced by some species of *Aspergillus* and *Penicillium,* particularly *Aspergillus ochraceus*, *A. carbonarius*, *A. niger* and *Penicillium verrucosum*. These moulds can easily contaminate foodstuffs, although the occurrence of OTA in foods may depend on climatic conditions [1].

Toxicity of OTA is well documented in many animal species, including human beings [2]. It is probably related to the Balkan endemic nephropathy (BEN), and the International Agency for Research on Cancer [3] has classified OTA as Group 2B, a possible human carcinogen. Exposure to OTA is worldwide, as known by its detection in human serum or urine of people from many countries [4].

OTA occurs predominantly in cereal grains, cereal products, cocoa, spices, oilseeds, coffee beans and legumes. However, cereal products are the major group of food commodities where the toxin is of greatest impact. The frequency of food contamination with OTA represents an important source of daily OTA intake, with consequences to human health [5]. Consequently, the European Union Scientific Committee for Human Feeding deemed necessary to take prudential measures in order to reduce the degree of exposure to OTA to levels below 5 ng/kg body weight (b.w.)/day [6].

The bread is a product of daily consumption and highly demanded; thus several authors have indicated bread as one of the main sources of daily intake of OTA [7–9]. The presence of OTA in bread results from the contamination of wheat flour, and probably only partly is destroyed during the bread making process [10].

Maize bread is a traditional and special type of bread very appreciated in Portugal, and it is consumed mainly in the North and Central Zone of country. This bread is made with cereals such as maize (*Zea mays*) and wheat (*Triticum aestivum*), where the ochratoxigenic moulds *A. ochraceus* and *P. verrucosum,* respectively, grows.

In Portugal, the agricultural production primarily consists in 1,425,000 tons of cereals such as rice (146,000 tons), maize (780,000 tons), wheat (300,000 tons) and barley (10,000 tons) [11]. According to the FAO, the consumption of cereal in

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Portugal was 1,335,000 tons in 2002, and according to IACA was 152,7000 tons. The human consumption increased in the following order: barley, maize, rice and wheat [12].

Few analytical methodologies for OTA determination in bread have been reported. González et al. [13] used pressurised liquid extraction with methanol, and liquid chromatography with fluorescence detection (LC–FD) for determination. Frequently for cereals and derivatives, the common extraction methodologies are based on the solubility of OTA in organic solvents. In this way it has been used liquid extraction with different solvents such as chloroform [14,15], methanol [1,16,17] or methanol with aqueous solution bicarbonate [18,19], and acetonitrile/water mixture [20,21]. For the sample preparation and clean-up, immunoaffinity columns (IAC) [19,20], ion exchange columns [21], matrix solid phase dispersion with C_8 and C_{18} (MSPD) [22,23], solid phase microextraction (SPME) [24,25] and molecularly imprinted solid phase extraction (MISPE) [15] have been used. IAC clean-up is widely used, allowing lower limit of detection compared to MSPD clean-up with C_{18} and accurate and reproducible results [23,26]. For detection and quantification of OTA and mycotoxins in general, the methods are based on thin-layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA) [27], liquid chromatography with electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS) [21] and mainly liquid chromatography with fluorescence detection (LC–FD) [19,20].

As far as we know, bread and maize bread, very much consumed in Portugal, have never been evaluated regarding OTA contamination. Furthermore, even for other countries only few papers reported OTA levels in wheat bread [10,13].

The objective in the present study was to optimize a sensitive and accurate method for determination of OTA in the maize bread samples by LC–FD, and providing data on the occurrence of OTA in 15 maize samples consumed in the central zone of Portugal.

2. Experimental

2.1. Apparatus

A Moulinex blender 700 W (230–240 V, 50–60 Hz) (Barcelona, Spain), a Braun MR 5000 M multiquick/minipimer 500 W (220–230 V, 50–60 Hz, Esplugues del Llobregat, Spain), an Ultra-Turrax homogenizer Ystral Gmbh Drive X10/25 (230 V; 50/60 H, Dottingen, Germany), a vacuum manifold of Macherey–Nagel (USA), a pump of Dinko (mod. D-95, 130 W, 220 V), a centrifuge (Meditroni S-599, Selecta, Barcelona, Spain), a magnetic stirrer (Agimatic-S, Selecta, Barcelona, Spain), a Retsh vortex mixer (Haan, Germany), and a Sonorex RK 100 ultrasonic bath (Berlin, Germany) were used.

The LC apparatus used consisted of a pump (Model 307, Gilson Medical Electronics, Villiers-le-Bel, France), one 50 μL Rheodyne injector (mod. 7125, Cotati, CA, USA), a guard column Hichrom Ltd., HI-173 (30 mm × 4 mm i.d.) (England), and a column Hichrom C_{18} (5 μm, 250 mm × 4.6 mm i.d.). A Perkin-Elmer spectrofluorimeter (Model LS 45, Perkin-Elmer, Beaconsfield, UK), operating at an excitation wavelength of 333 nm and an emission wavelength of 460 nm, was used. The spectral bandwidth was 10 nm for both excitation and emission. The mobile phase (acetonitrile/water/acetic acid 49.5:49.5:1.0, v/v/v) was maintained at a flow rate of 1 mL/min.

The results were recorded on a 3390A integrator (Hewlett-Packard, Philadelphia, PA, USA).

2.2. Chemicals

Filter paper Whatman N° 4 (150 mm Ø, Whatman International Ltd. Maidstone England) was used.

LC grade acetonitrile, methanol and toluene were purchased from Carlo Erba (Milan, Italy). Acetic acid, hydrochloride acid, sodium hydroxide, potassium chloride, potassium dihydrogenphosphate, anhydrous disodium hydrogenphosphate, and sodium chloride analytical grade were obtained from Merck (Darmstadt, Germany). Water was purified by distillation and passage through a Milli-Q system (Millipore, Bedford, MA, USA). OTA was purchased from Sigma Chemical Co. (St. Louis, MO, USA) with purity grade ≥98%. Boron trifluoride–methanol (14% solution) was obtained from Sigma Chemicals Co (St. Louis, USA).

A standard solution of OTA was prepared from the OTA vial purchased from Sigma. The standard stock solution was made in 4 mL toluene:acetic acid (99:1) at 250 μg/mL, and stored at −20 °C. An intermediate standard solution was prepared at 10 μg/mL, by diluting 1 mL of stock standard solution with 25 mL toluene:acetic acid (99:1).

For fortification assays, a standard solution was prepared in toluene:acetic acid (99:1) at 1 μg/mL and two work solutions with mobile phase at 0.025 and 0.1 μg/mL. For the calibration curve, standard solutions were prepared by evaporating 100 μL of intermediate standard solution to dryness and diluting to 10 mL with mobile phase (0.1 μg/mL). After suitable dilutions in water:methanol:acetic acid (49.5:49.5:1), the working standard solution was used to prepare solutions at10 and 5 ng/mL, and 25 μL were injected.

Phosphate buffer solution (PBS) was prepared from potassium chloride (0.2 g), potassium dihydrogen phosphate (0.2 g), anhydrous disodium hydrogen phosphate (1.2 g), and sodium chloride (8 g) added to distilled water (900 mL). After dissolution, the pH was adjusted to 7.4 (with 0.1 M HCl or 0.1 M NaOH as appropriate), and the solution was made to 1 L.

IAC Ochratest columns (Vicam, Watertown, MA, USA) were used for clean-up.

All chromatographic solvents and water were degassed for 15 min in ultrasonic bath. Decontamination of the glassware was performed using a sodium hypochlorite solution. It was then acid-washed by immersing the glassware in a solution of 4 mL/L H_2SO_4, and then washed to neutral pH by rinsing with distilled water.

2.3. Sampling

A total of 15 samples were purchased in commercially available size during September 2005 from bakeries, confectionery's shops and supermarkets located in the city of Coimbra and its

countryside, central zone of Portugal. The samples were transported to the laboratory under ambient conditions. Samples were milled using a blender Moulinex.

All of the information about the samples was obtained from the labels. The milled samples were analysed as quickly as possible after the purchase. When this was not possible, they were stored at −20 °C.

2.4. Recoveries

For recovery studies, 26.5 μL of the OTA work solution prepared in mobile phase at 25 ng/mL, and 50, 100 and 400 μL of the OTA work solution at 100 ng/mL were added to 20 g maize bread, and allowed to stand for 15 min at room temperature before extraction, for three replications. Because no certified reference materials for bread were available, trueness was assessed through recovery of additions of known amounts of OTA. The fortification levels were 0.033, 0.25, 0.50 and 2.00 ng/g, respectively (Tables 1–4).

Table 1
Mean recoveries and R.S.D.s obtained ($n = 3$) with different volumes of PBS/methanol (50:50) as extracting solvent using Minipimer apparatus

PBS:methanol volumes	Fortification level (ng/g)	Recovery (%)	R.S.D. (%)
50:50 (100 mL)	0.5	102.0	4.7
50:50 (75 mL)	0.5	65.0	17.8
50:50 (50 mL)	0.5	51.0	6.0

Table 2
Mean recoveries and R.S.D.s obtained ($n = 3$) with different proportion of PBS/methanol as extracting solvent

PBS:methanol volumes	Fortification level (ng/g)	Recovery (%)	R.S.D. (%)
50:50 (100 mL)	0.5	102.0	4.7
80:20 (100 mL)	0.5	99.6	0.7

Table 3
Mean recoveries and R.S.D.s obtained ($n = 3$) between different agitation techniques using 100 mL PBS:MeOH (50:50) and OTA fortification level at 0.25 ng/g

Agitation (time)	Recovery (%)	R.S.D. (%)
Manual (15 min)	50.0	6.0
Ultra-Turrax (5 min)	53.0	3.2
Centrifugation (15 min)	30.0	5.8
Agitation plate (15 min)	22.0	5.0

Table 4
Accuracy and intra-assay validation results ($n = 3$) and inter-assay ($n = 3$) obtained with the optimized method

Fortification (ng/g)	Recovery (%)	R.S.D. intraday (%)	R.S.D. interday (%)
0.033	80.4	8.8	12.1
0.25	92.3	5.2	10.9
0.5	102.0	4.7	11.4
2.0	87.0	1.4	9.3

2.5. Sample extraction and clean-up

An aliquot of sample (20 g) was extracted with 100 mL PBS/methanol (50:50, v/v) using the Braun Minipimer homogeniser for 5 min; the mixture was filtered through a Whatman filter paper. After 20 mL aliquot of the filtered was diluted with 30 mL of PBS and then this solution was passed through an IAC column for clean-up using a vacuum manifold. The column was washed with 10 mL of water before eluting OTA with 3 mL of methanol. The methanol was dried at ±50 °C under a gentle nitrogen stream, and the residue was reconstituted in 250 μL of mobile phase by mixing, and 50 μL were injected in the LC system.

2.6. Chemical confirmation of OTA by methyl ester formation

For confirmation, OTA was converted into its methyl ester using boron trifluoride methanolic solution 14% (BF_3-CH_3OH 14%) [5,28]. Sample extracts were evaporated to dryness, 150 μL of the BF_3-CH_3OH 14% solution was added, and the mixture was left at 60 °C for 10 min. After evaporation, the residue was dissolved in 250 μL of mobile phase.

3. Results and discussion

The calibration curves were obtained using the linear least squares regression procedure of the peak area versus the concentration. The linearity for OTA, in the working standard solutions at three determinations of five concentration levels, between 1 and 25 ng/mL, was good as shown by the fact that the determination of the correlation coefficients (r^2) are above 0.9990 for six calibration curves, prepared in three different days.

To optimize the extraction of OTA from maize bread, the extraction efficiencies were studied in order to achieve good analytical performance (Fig. 1). Firstly, OTA was extracted using the method of Pena et al. [28], previously used for rice samples. However, some modifications were needed. Three different volumes of PBS:methanol (50:50) were assayed as extracting solvent, using Minipimer apparatus: 50, 75 and 100 mL by using 20 g of maize bread fortified with OTA at 0.5 ng/g. The best recoveries were obtained with 100 mL of PBS:methanol (50:50) (Table 1). The extracting solvent was proved in different proportions and recoveries obtained with PBS:methanol (80:20) were slightly lower, 102% versus 99.6% (Table 2). This phenomenon may be due to the solubility power of methanol [28,29]. Higher percentage of methanol were not studied because OTA was able to could be elute from IAC columns [28], since methanol is one of the most potent desorbents [15].

Due to the characteristics of the sample, a more efficient process to separate the matrix residue from the solvent extract was essential. So, firstly filter paper Whatman N°4 and secondly centrifugation at 3400 × g for 15 min, were evaluated, and it was observed that with the second approach the recovery was 30%.

During the optimization of the method, different extraction procedures were assayed such as manual agitation, Ultra-Turrax,

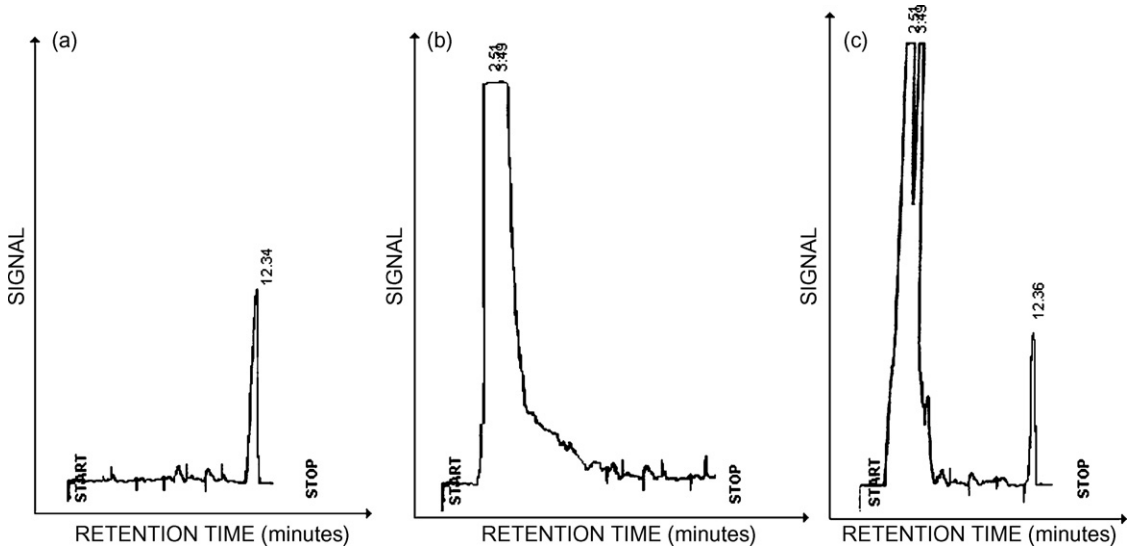

Fig. 1. LC-spectrofluorimeter chromatograms of: (a) solution of OTA standard at 10 ng/mL; (b) a maize bread blank sample; and (c) a maize bread sample fortified with 0.5 ng/g.

centrifugation and agitation plate. With these apparatus the recovery results oscillated between 22% and 53% (Table 3), which were overcome by Minipimer apparatus.

The accuracy for the optimized methodology was determined by calculating the mean recovery values used for each fortification level (Table 4). The recovery values oscillated between 102.0% and 80.4% for fortification levels at 0.5 and 0.033 ng/g, respectively. The precision was calculated through intraday repeatability ($n = 3$) and interday repeatability (3 days). The intraday repeatability was between 8.8% and 1.4% for 0.033 and 2.0 ng/g fortification levels, respectively. The interday repeatability was between 12.1% and 9.3% for the same fortification levels.

The LOQ was determined by the signal-to-noise approach, defined as that level resulting in a signal-to-noise ratio of approximately 10:1. The LOQ of the method was 0.033 ng/g. This value is lower than the obtained by González et al. [13] using pressurized liquid extraction with methanol for the analysis of OTA in bread, 0.06 ng/g.

3.1. Application to real samples

The optimized method of OTA extraction in maize bread followed by LC–FD was applied for the OTA analysis in 15 maize bread samples (Fig. 2). The frequency and incidence obtained are shown in Table 5. The frequency of OTA in analysed samples was

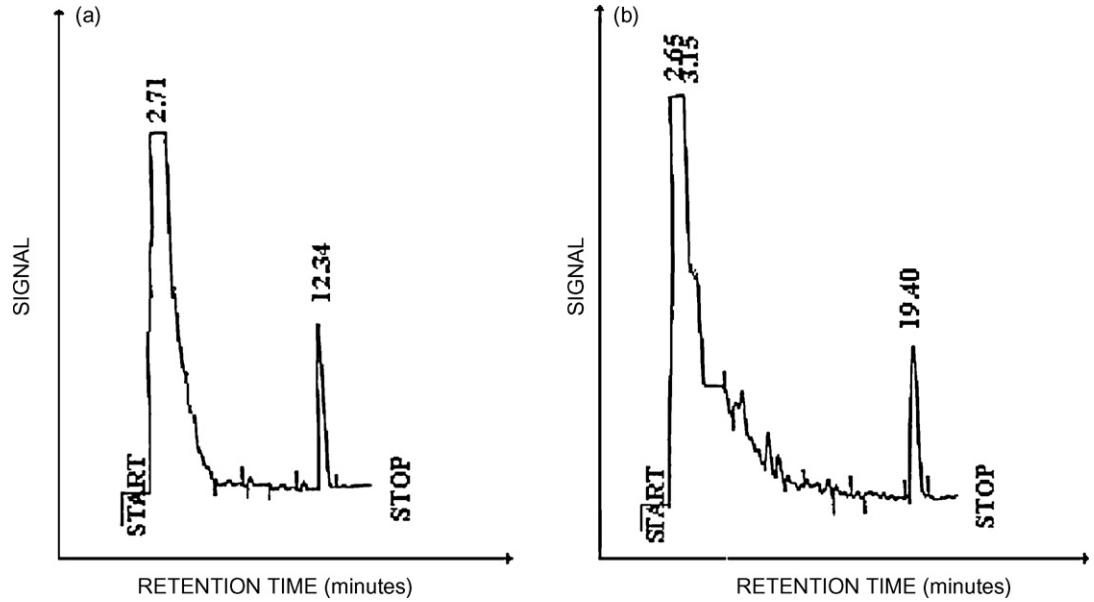

Fig. 2. LC-spectrofluometric chromatograms of: (a) a positive maize bread sample with 0.87 ng/g and (b) OTA methyl ester after boron fluoride–methanol derivatization.

Table 5
Prevalence and levels of OTA in maize bread

No of analysed samples	15
No of positive samples	9
Frequency (%)	60
<LOQ	1
Range (ng/g)	n.d.-2.65
Mean ± S.D. (ng/g)	0.43 ± 0.9
Median (ng/g)	0.08

60% (nine positive samples). The OTA contaminated samples revealed levels ranging from 0.033 and 2.65 ng/g and were being the OTA mean level 0.43 ± 0.9 ng/g and the median 0.08 ng/g. Among the nine positive samples, one presented an OTA level of 2.65 ng/g, which is near the maximum permitted level of OTA in cereal products established by the EU Commission Regulation, 3 ng/g, [30].

Few studies about OTA levels in bread samples are available. González et al. [13] found two contaminated samples in 20 bread samples analysed in Spain with OTA levels of 2.55 and 1.82 ng/g. Legarda and Burdaspal [10], in 255 samples of wheat bread from different countries, found 100% of incidence. The mean OTA levels ranged from 0.08 ng/g Austrian samples to 0.45 ng/g Spanish samples. The highest OTA level was found in one Spanish sample with 7.37 ng/g.

In the contaminated samples, the presence of OTA was confirmed by methyl ester formation with boron trifluoride in methanol (14%), followed by LC analysis. With this derivation the methyl ester was estimated to be 93% [28], therefore this reaction was not used for quantitative purposes.

4. Conclusions

Extraction with PBS/methanol, filtration and dilution with PBS allows the supernatant to be applied onto the IAC column, making it possible to achieve low limits of detection. This optimized analytical methodology provides good results in terms of accuracy, repeatability, intermediate precision and sensitivity, and has shown to be reliable for determination of OTA in maize bread, presenting limits of detection of 33 ng kg^{-1}.

The application of the procedure to 15 samples from the central zone of Portugal has demonstrated that 60% were contaminated, although none of the samples exceeded the recommended limit.

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